The scarless heart and the MRL mouse

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The ability to regenerate tissues and limbs in its most robust form is seen in many non-mammalian species. The serendipitous discovery that the MRL mouse has a profound capacity for regeneration in some ways rivalling the classic newt and axolotl species raises the possibility that humans, too, may have an innate regenerative ability. The adult MRL mouse regrows cartilage, skin, hair follicles and myocardium with near perfect fidelity and without scarring. This is seen in the ability to close through-and-through ear holes, which are generally used for lifelong identification of mice, and the anatomic and functional recovery of myocardium after a severe cryo-injury. We present histological, biochemical and genetic data indicating that the enhanced breakdown of scar-like tissue may be an underlying factor in the MRL regenerative response. Studies as to the source of the cells in the regenerating MRL tissue are discussed. Such studies appear to support multiple mechanisms for cell replacement.

Keywords: regeneration; MRL mouse; cryo-injury; MMPs; heart; scarring

1. THE MRL MOUSE, ITS HISTORY AND ITS UNUSUAL RESPONSE TO INJURY

The MRL mouse was generated through the interbreeding of the LG mouse (75%; H-2d/f), the AKR mouse (12.6%; H-2k), the C3H mouse (12.1%; h-2k) and the C57B1/6 mouse (0.3%; H-2b) (Murphy & Roths 1979). The MRL.*lpr*/*lpr* mouse was selected originally for its large size and was found to have a major defect in immune regulation. It was found that with age, lymphocytes in the lymph nodes and spleen showed unregulated proliferation (thus lpr), which could be easily seen at visual inspection of the mouse as lumps under the skin. This phenotype was found to be due to a single gene, the *fas* gene, a gene involved in cell death. The *lpr* mutation was then shown to be due to a retrotransposon insertion into the second intron of the *fas* gene (Watanabe-Fukumaga *et al.* 1992; Adachi *et al.* 1993) and led to an absence of cell death. This lpr mutation resulted in multiple autoimmune effects including aberrant control of apoptosis in germinal centres, autoimmune sequelae, autoantibodies, and an arthritis-like syndrome and has been used extensively as a model of lupus erythematosis.

Using this mouse as an autoimmune model, we found that the MRL mouse displayed yet another remarkable capacity and that was for tissue regeneration. Young adult autoimmune MRL.*lpr*/*lpr* as well as normal nonautoimmune MRL.*lpr*/+ (MRL/MpJ) mice were found to be capable of completely closing 2 mm through-and-through surgical ear holes within 30 days, whereas all other mice tested have residual open holes, this being a standard animal colony numbering technique that is usually stable over the lifetime of the animal (figure 1; Desquenne-Clark *et al.* 1998; Heber-Katz 1999). The healing seen in these MRL mice displays normal gross and microanatomic tissue architecture reminiscent of regeneration seen in amphibians (Gross 1996; Stocum 1996; Brockes 1997), whereas the normal outcome in mammals is scarring (Clark 1996). In fact, the type of healing seen is mechanistically different.

Although there are no dramatic examples of regeneration in mammals, it has been shown that antler regrowth (Goss 1970; Allen *et al.* 2002; Price & Allen 2004) and fingertip regrowth (Borgens 1982) does occur. In fact, it has been shown that ear holes in rabbits as well as cats close and regrow cartilage (Goss & Grimes 1975).

2. THE GENETICS OF REGENERATION

Both the healer MRL mouse as well as the control nonhealer C57BL/6 (B6) mouse used in most of our experiments are inbred strains. A cross between an MRL and B6 or the F_1 intercrossed population showed intermediate healing and F_1 intercrosses to produce F_2 populations were also intermediate healers. We used these as well as backcross populations to initially map the genetic *heal* loci involved with the ear-hole closure regenerative response using microsatellite markers. Initial experiments showed the involvement of seven loci (McBrearty *et al.* 1998). Importantly, none of the heal loci was shown to overlap with the autoimmune loci that had been previously mapped and that included the *fas* gene as well (Watson *et al.* 1992). Further crosses and the use of nonhealer strains other than the B6 have revealed an even more complex picture with at least 20 different loci involved in this response (table 1; Masinde *et al.* 2001; Blankenhorn *et al.* 2003; Heber-Katz *et al.* 2004). We also developed congenic mice made by multiply backcrossing the healing MRL mouse strain to the nonhealing B6 strain and

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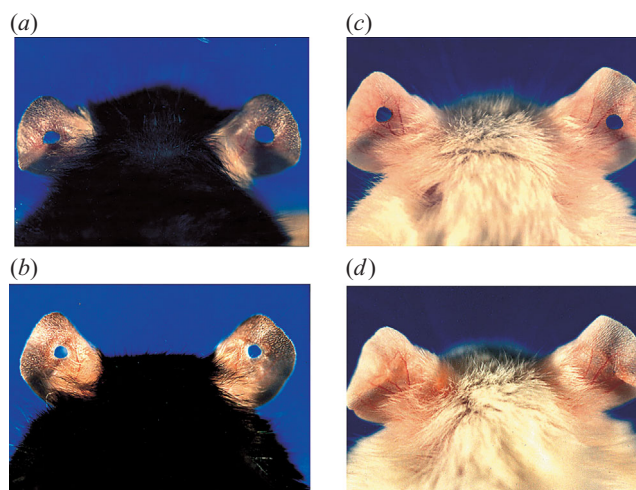DOI 10.1098/rstb.2004.1468

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Figure 1. Ear-hole closure after 30 days. Through-and-through 2 mm holes are punched in the middle of the ear pinnae in both MRL (*a,b*) C57BL/6 (black mouse) and (*c,d*) MRL (white mouse) ears. The holes can be clearly seen in the C57BL/6 at days 0 (*a,c*) and 30 (*b,d*). However, by day 30 in the MRL (*d*) the holes have disappeared.
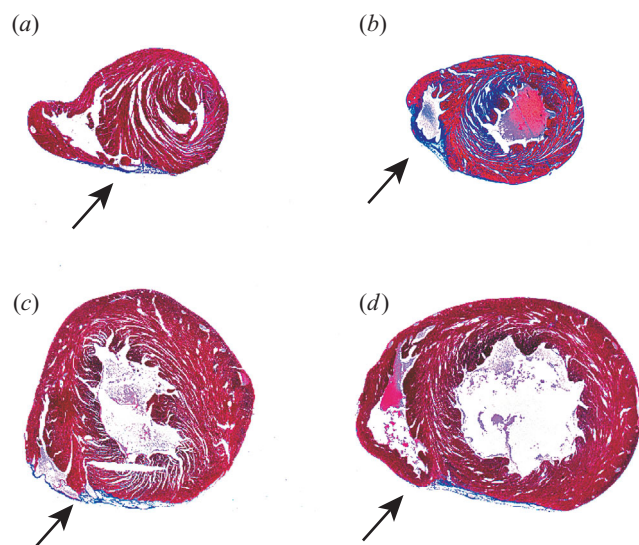

Figure 2. MRL myocardium regenerates. Histological sections of the mouse heart are stained with trichrome and the collagen is stained in blue. Five to 7 days after cryo-injury to the RV of the heart, the injury can be seen in both the C57BL/6 (*a*) and MRL (*b*) hearts. By 60 days, an acellular scar can be seen in the C57BL/6 (*c*) and near complete myocardium has filled the injury site in the MRL (*d*) heart.

selected the mice for those displaying rapid healing. Such mice were still multigenic, confirming many of the loci demonstrated by microsatellite mapping, and thus further suggesting that ear-hole closure was a complex genetic trait (L. Clark and E. Heber-Katz, unpublished data).

In studying ear-hole closure, we found that in both the MRL and B6 strains, male and female mice healed ear holes differently, showing that the ear-hole closure response was sexually dimorphic. This was further demonstrated in that the genetic loci that had been mapped were associated with the male, female or both populations (Blankenhorn *et al.* 2003).

3. THE HEART

The heart is an organ not considered to regenerate in mammals. This is in contrast to the studies showing a regenerative heart response in non-mammalian vertebrates both *in vivo* and *in vitro* (Becker *et al.* 1974; Carlsten *et al.* 1982; Flink 2002; Poss *et al.* 2002; Bettencourt-Dias *et al.* 2003). Indeed, the scarring response following myocardial infarction is the major predicate to chronic heart disease and death in humans. Using a freezing injury model that ensures cell death in a given area of tissue, we made a cryo-injury to the RV of the heart through the diaphragm using both MRL and B6 mice (figure 2). By day 5 in both strains of mice, the cryo-injured area was full of fibrotic cells which had replaced the cardiomyocytes. By day 60, the MRL showed normal myocardium and little or no scarring whereas the B6 mouse showed an acellular scar which had replaced the myocardium (Leferovich *et al.* 2001).

To follow the injury made in a given animal over time, we carried out echocardiography several days after injury and then monthly. The MRL mice showed an enlarged RV, which returned to its normal size over a period of three months. Furthermore, histological examination of the hearts after 1 year showed a normal MRL heart compared with a nonhealed and scarred B6 heart (Leferovich *et al.* 2001; Leferovich & Heber-Katz 2002).

How does this type of healing occur in the MRL? It is generally held that adult mammalian cardiomyocytes are terminally differentiated and do not re-enter the cell cycle (Nadal-Ginard 1978; Soonpaa & Field 1998; Taylor *et al.* 2002), though reports suggesting otherwise have been attributed to stem cell populations (Urbanek *et al.* 2003; Beltrami *et al.* 2003). Thus, one possible explanation for our results is that cardiomyocytes have migrated into the injury site in the MRL. A second possible explanation is that stem cells are responsible for such replacement.

To begin to address the above issues, we examined a cell-cycle-associated molecule that is transiently expressed, Ki-67. We found that early in the injury response, specifically between days 7 and 15 after injury, cardiomyocytes in the MRL expressed Ki-67 (K. Bedelbaeva, J. Leferovich and E. Heber-Katz, unpublished data).

As Ki-67 is expressed only during cell division, we could only determine at given time points what was happening at that particular time point. To determine the total number of cells that were dividing over the 60 day period, we used the molecule BrdU, a nucleic acid analogue, a marker of cell division, and a molecule that is incorporated into DNA and can be detected using a specific antibody. BrdU was given to the mice during the full healing period so that the BrdU would continue to be incorporated and would not become diluted. At 60 days, after most of the healing was seen, we examined the tissue for BrdU incorporation into cardiomyocytes and specifically that quadrant of a cross-section of the heart that was injured. We found that large numbers of cardiomyocytes were BrdU positive, *ca.* 20% compared with the B6, which showed 2% labelling. Of course, these experiments did not provide information about the source of the cells involved in the healing. Such possibilities included the division of mature adult cardiomyocytes, the transdifferentiation of other mature cell types into cardiomyocytes, or the

Table 1. Heal QTL in all strains.

(*Heal* designations are as seen in McBrearty *et al.* (1998), Blankenhorn *et al.* (2003) and Heber-Katz *et al.* (2004). *Sth* designations are as seen in Masinde *et al.* (2001).)

peak marker	cM	name of healing QTL	origin of healer in MRL × B6	origin of healer in MRL × CAST
D8Mit211*	49	*Heal1*	B6	
D13Mit15,16	9–13	*Heal2*	MRL	MRL
D13Mit228	60–65	*Heal3/Sth10*	MRL	
D15Mit244	57	*Heal4*	MRL	
D12Mit136	13	*Heal5*	B6	
D7Mit85	27–52	*Heal6*	MRL	
D13Mit245- D13Mit139	30–36	*Heal7*	MRL	MRL
D4Mit148	66–71	*Heal8*	MRL	
D18Mit123	30–44	*Heal9*	B6	
D11Mit213- 61	58–70	*Heal10*	MRL	MRL
D16Mit122-110	4–20	*Heal11*	het	
D14Mit233	20	*Heal12*		CAST/Ei
D17Mit93	44	*Heal13*		CAST/Ei
D1Mit334	49.7	*Sth1*		
D3Mit217	nd	*Sth2*		
D4Mit214	18	*Sth3*		
D4Mit31	51	*Sth4*	MRL,	MRL
D6Mit261	37	*Sth5*		
D7Mit220	52	*Sth6*	BC1 suggestive	
D7Mit12	66	*Sth7*		
D9Mit207	33	*Sth8*		
D9Mit270	43	*Sth9*		Het
D2Mit354	0–5	suggestive		Het

*D8Mit22 is DNA segment, Chr. 8, MIT211.

transdifferentiation of stem cells, whose involvement has begun to be examined as discussed in § 4.

4. THE SCAR RESPONSE

In addition to the presence of dividing cells, the lack of scar in the MRL was quite striking as compared with the control mouse, the B6. The lack of a scar after injury is not totally foreign to mammals. This, however, is found in early development where it has been reported that foetal animals heal without scar tissue (Armstrong & Ferguson 1995; Ferguson & O'Kane 2004). The foetal wound area develops hair follicles and has normal tissue architecture and normal collagen superstructure. These are elements that would be ascribed to regeneration in adults. By contrast, adults usually heal with scar formation in which there are no newly developing hair follicles, there is abnormal collagen structure and abnormal tissue architecture and function. For instance, skin wound scars are weaker and heal poorly upon re-wounding.

There is an age-related developmentally directed component to this transition as well. Experiments done with marsupials show that the transition point between no scarring and scarring occurs at pouch day 9. Evaluation of the factors at this time point reveal an obvious inflammatory response which appears in ontogeny at day 9. It was concluded that an immune response was probably involved (Ferguson *et al.* 1996). In foetal mice, the transition point is found to be embryonic day 16, coincident with the appearance of inflammatory responses to wounds in mouse ontogeny (Hopkinson-Woolley *et al.* 1994). Interestingly, this is also the point that T cells with

rearranged αβ TcR begin to appear in the thymus (Havran & Allison 1988). From these studies, it is suggestive, then, that T cells play a pivotal role in the outcome of a neonatal wound. We have begun to address this issue and have preliminary data suggesting that T cells from nonhealer mice do inhibit the ear-hole closure response.

5. THE MECHANISMS INVOLVED IN THE MRL REGENERATIVE RESPONSE

Perhaps one of the most telling aspects of ear-hole closure in the MRL mouse is seen in the ability of this mouse to break down its own basement membrane that forms during and after epidermal coverage of the wound. With the usual repair mechanisms generally associated with mammalian wound healing, including the formation of a provisional matrix and a remodelling response, a basement membrane forms and separates the epidermis from the dermal layer. However, in the regenerative response seen in amphibians, the basement membrane either never forms or does so and disappears so quickly that it has not been reported to be present. The growth of the blastema soon follows (Stocum & Dearlove 1972; Globus *et al.* 1980; Brockes 1997). The importance of the basement membrane and its presence or absence has been demonstrated by the induction of a basement membrane in the amphibian limb stump. This event leads to the cessation of the regenerative response and to the formation of a scar (Stocum & Crawford 1987).

Looking at the healing ear, we noted that the kinetics of ear-hole closure showed a dramatic shift of the healing curve on day 5 in the MRL. We thus examined the

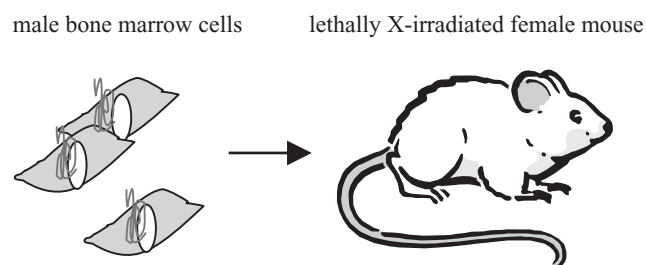male bone marrow cells   lethally X-irradiated female mouse

Figure 3. Generation of bone marrow chimeras. The source of the dividing cells. Radiation bone marrow chimeras were made by lethally irradiating 10-week-old MRL and C57BL/6 female mice (900 rad from a ^{137}Cs gamma-ray source). Bone marrow cells from male adult mice were then injected intravenously and animals were put on antibiotics. Thirty to 60 days later, the mice were used for ear-hole punching or cryo-injury to the heart.
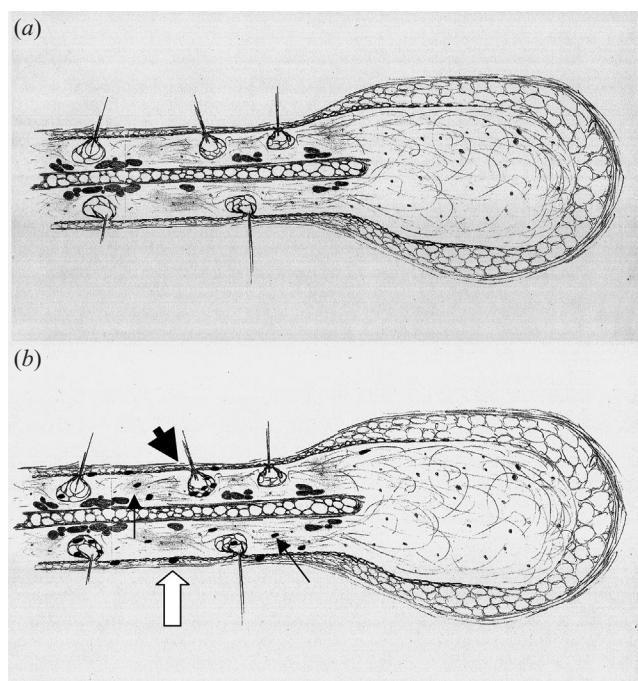

Figure 4. MRL male/female chimeric ear-hole blastema. A drawing of the histological and *in situ* hybridization results can be seen. MRL chimeric ears were hybridized using a Y-chromosome DNA probe that was digoxigenin labelled, treated with a horseradish peroxidase-labelled anti-digoxigenin antibody, and then treated with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-5-yl phosphate. In (*a*), a control *in situ* hybridization showed no staining. In (*b*), specific Y-chromosome *in situ* hybridization (blue) could be seen in the dermis (black arrows), in the hair follicles (arrowhead), and in the epidermis (white arrow).

epidermal–dermal extracellular matrix structure at time points around this event. We found that on day 4, a clear basement membrane had formed between the epidermis and dermis in both the MRL and B6 ears. However, a day later, the basement membrane had disappeared in the MRL and was still not seen at least up to day 7. In the B6, however, the basement membrane formed and remained throughout (Gourevitch *et al.* 2003).

Though a similar response was not seen in the heart because the tissue architecture is different, we did examine the collagen protein response to injury. Here, we found that the level of collagen as measured by the hydroxypro-line levels in the B6 went up after injury, consistent with the formation of the scar. By day 60, that level returned to normal. In the MRL, however, the level of collagen was twofold higher than the B6 and after injury the level went down continuously until day 60. This reduction in protein levels was striking and was not reflected in the collagen type I RNA levels where both the MRL and B6 hearts responded to injury by showing enhanced RNA synthesis by day 5, but normal RNA levels by day 15 (Leferovich *et al.* 2001). These results suggested to us that there was a protease that was degrading the collagen in the MRL heart.

6. THE MOLECULES INVOLVED IN THE MRL REGENERATIVE RESPONSE

A group of molecules shown to be involved in both wound repair and regeneration, and specifically in a remodelling response involving collagen as well as other extracellular matrix molecules, is the family of MMPs (Grillo *et al.* 1968; Matrisan 1992; Yang & Bryant 1994; Parks 1999; Chernoff *et al.* 2000; Quinones *et al.* 2002). This is especially true for two family members, MMP-2 and MMP-9. There are multiple cell types that produce and secrete these proteases.

In the case of ear-hole closure, we found that both molecules were differentially expressed between MRL and B6. We also found that most of these molecules are expressed by inflammatory cells and are brought into the injury site soon after wounding. Thus, neutrophils that rise and fall at *ca.* 2–3 days after the injury are positive for both

MMP-2 and MMP-9. Also, macrophages that peak a few days later are also MMP-2 and MMP-9 positive. Though the inflammatory response is greater in the MRL than in the B6 after ear punching, the level of MMP-positive cells in the MRL compared with the B6 is even more exaggerated. The antibodies used to detect the MMPs in cells recognize both the pro and active form of MMP. Zymography, a technique that combines gel electrophoresis and enzymatic breakdown of given proteins, allows the separate analysis of these two forms. Using this, we found that there is also more active form of the MMPs in the MRL than in the B6 healing ear blastemas.

A family of molecules that inhibit the MMPs, i.e. TIMPs, are also expressed during healing. Those TIMPs that are specific for MMP-2 and MMP-9 are also expressed at different concentrations in the MRL and B6 ear-hole response. In this case, the TIMP levels are lower in the MRL than in the C57BL/6 (Gourevitch *et al.* 2003). Thus, the MRL has increased amounts of active MMPs and lower amounts of inhibitor, together promoting a more vigorous protease response.

Examination of the heart and the RV injury over time for MMP expression showed similar results to the ear. Again, MMP-2 and MMP-9 could be found in the inflammatory cells and more specifically in the neutrophils. And, unlike the ear, in the heart the neutrophils persisted. Here, MMP-2-positive cells were still present in the MRL heart by day 15 though they were gone by day 10 in the B6
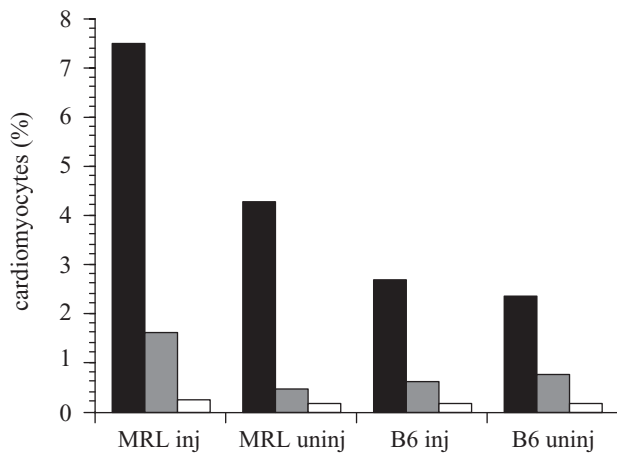
Figure 5. The incorporation of BrdU or Y-chromosome markers into cardiomyocytes after injury. Syngeneic chimeric mice were created by injecting male bone marrow into lethally X-irradiated female mice to determine the level of bone marrow contribution to the normal or dividing cardiomyocyte population after heart cryo-injury. Male cells were followed by using *in situ* hybridization with a Y-chromosome digoxigenin-labelled probe as described in figure 4. Dividing cells were determined by BrdU incorporation and detected using an anti-BrdU antibody and a secondary FITC-labelled antibody. Animals were continually given BrdU in the drinking water from the time of cryo-injury to the end of the experiment. The identification of double-stained cells or male cells that were dividing was done by using the above two labellings together. The groups shown are chimeric MRL and B6 mice either uninjured (MRL uninj; B6 uninj) or cryo-injured (MRL inj; B6 inj). Data are presented as percentages of total cardiomyocytes and were determined by counting labelled cardiomyocytes and comparing with the total number of cardiomyocytes in that histological section. Black bars, BrdU positive; grey bars, Y positive; white bars, double positive.

heart. Also, MMP-9-positive cells were still rising by day 7 in the MRL heart but disappearing in the B6 at that same time point (K. Bedelbaeva, unpublished data).

The MMP experiments described herein did show a correlation between the greater level of MMPs, the lower level of TIMPs in the MRL, the basement membrane breakdown, and blastema formation. However, to prove this relationship, we used an MMP inhibitor to show its requirement. The antibiotic minocycline specifically blocks MMP activity (Ohishi *et al.* 1995). We treated mice before and up to 10 days during ear-hole closure. The MRL mice showed a significant reduction in ear-hole closure at day 30 with a *ca.* 1 mm diameter hole instead of a 0 mm diameter hole. No effect was seen in the B6 mice. With similar treatment, the heart injury in the MRL also showed less healing (L. Clark, J. Leferovich and E. Heber-Katz, unpublished data).

It is interesting to note that the remodelling response in the mammalian heart after injury is generally considered to add to heart failure (Lee 2001; D'Armiento 2002; Death *et al.* 2002; Lindsey *et al.* 2003). On the other hand, the MMPs and specifically MMP-9 have been shown to be involved with the scarless healing response seen in foetal animals (Peled *et al.* 2002).
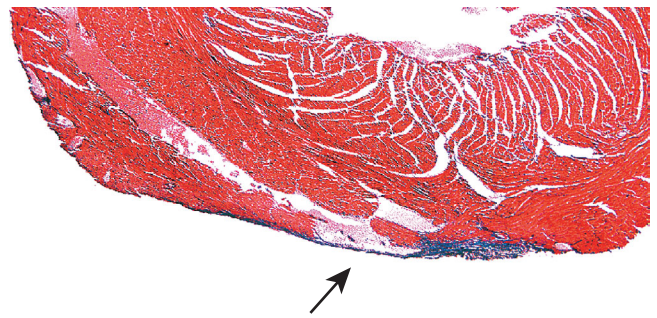
Figure 6. Healer into nonhealer chimeric myocardium shows nonhealer-type healing. The heart seen is derived from a chimeric mouse which was created by the injection of F_1 bone marrow into a lethally X-irradiated (900R) C57BL/6 mouse. The histological section of the mouse heart 30 days after cryo-injury is stained with trichrome with collagen staining blue. The arrow shows the acellular scar typical of C57BL/6 healing (the injury was less severe than usual). Alternatively, the level of collagen produced may be more similar to early MRL-like healing.

7. THE CELLS THAT CONTRIBUTE TO THE REGENERATIVE RESPONSE

Looking at the healing of wounds and the growing of new tissue in the MRL mouse does not give us any information about where the cells involved in this response come from. To answer such a question, one needs to label cells and introduce them into the animal and then ask, after wounding and healing, which of those cells are labelled. Such experiments have been carried out using mature cells (Lo *et al.* 1993; Echeverri *et al.* 2001; Brockes & Kumar 2002). But it is also possible to use stem cells such as bone marrow-derived cells to follow the contribution of these cells to the wound (Fuchs & Segre 2000).

We did several experiments to examine the issue of (i) which cells in the ear make up the regeneration blastema and the healed ear tissue or (ii) which cells in the healed heart after the cryo-injury-induced tissue damage come from local tissue or come from an immature circulating population of cells. To do this, we generated animals with haematopoietic and other stem cells of one type and stromal cells of another type. Thus, we generated bone marrow chimeras using female mice as the recipients and bone marrow cells from male mice. Before injection of cells, the female mice were lethally X-irradiated and 1 day later injected with the male bone marrow-derived cells (figure 3). After 30–60 days, an injury was made and, after another 30 days, the tissue was examined. The use of male cells into female mice is based on the fact that male cells all have the Y chromosome and this can be detected by using a Y-chromosome nucleic acid probe that hybridizes to the Y-chromosome DNA. In addition, to prove that the male cells survived and repopulated the female mice, we examined tissue from the spleen and the gut.

In the first set of experiments, we made syngeneic chimeras by injecting male bone marrow cells from healer MRL mice into lethally X-irradiated healer MRL female mice and by injecting nonhealer B6 male bone marrow into lethally X-irradiated nonhealer B6 female mice. In carrying out these experiments, we first showed that even after lethal irradiation of the host and then reconstitution with

bone marrow, these animals still healed according to their original phenotype. Thus, the MRL chimeric mice were ear-hole healers and the B6 chimeric mice were ear-hole nonhealers.

Thirty days after ear punching both the MRL and B6 syngeneic bone marrow chimeras, we examined the ear holes. Chimerism was shown to be positive in both the gut and the spleen in these mice. In our preliminary studies (30 days between chimera creation and wounding) using the MRL chimeras, we found that ear tissue distant from the injury site had been repopulated extensively with Y-chromosome-positive cells in the hair follicles and the basal epidermal cells. However, when we looked at the blastema region directly at the wound site in ear holes that had almost but not completely closed, we saw *no* Y-chromosome hybridization, though previous experiments have shown that these cells are clearly dividing (figure 4). If we now waited 60 days after creating the chimeras and again 30 days after ear punching, we saw many more Y-chromosome-positive cells and in this case Y-positive cells were found in the blastema and more so in the new hair follicles. The B6 chimera results showed little Y-chromosome-positive cells post-wounding even though chimerism in the gut was seen. This indicated that though bone marrow-derived cells can contribute to the blastema and in particular the hair follicles and epidermis, this is not necessarily the case. It must be that there is another source of cells that contributes to the increase in cell number through cell division in the MRL mouse ear, previously shown using BrdU incorporation (K. Bedelbaeva, B. Freedman, L. Clark and E. Heber-Katz, unpublished data). One possibility is the presence of an immature host-derived (female) population that, of course, does not label with the Y-chromosome probe. However, another possibility is that the MRL mouse forms a blastema from mature host (female) tissue like the newt, i.e. through the de-differentiation of muscle and cartilage present in the limb (Brockes 1997; Tanaka 2003).

This result is consistent with a previous mouse ear-hole closure study (Kench *et al.* 1999) in which nonhealer B10.BR bone marrow into healer MRL recipient chimeras and healer MRL bone marrow into nonhealer B10.BR recipient chimeras displayed the healing phenotype of the recipient, leading the authors to conclude that healing was due to a nonhaematopoietic, radioresistant cell type of the host.

Using the syngeneic chimeras already mentioned, we examined these mice for their healing phenotype after a cryo-injury to the RV of the heart. Previous studies have shown that bone marrow-derived cells do contribute to the cardiomyocyte population in the heart (Ferrari *et al.* 1998; Jiang *et al.* 2000; Orlic *et al.* 2001*a*,*b*; Jackson *et al.* 2001; Goodell *et al.* 2001; Toma *et al.* 2002). In our experiment, 30 days after creating the chimeras and 30 days after injury, we saw healing without scarring in the MRL and healing with scar formation in the B6 injury site. Like ear-hole closure, the heart healing phenotype was the same as that seen in normal mice, with the MRL chimeras displaying healing with new cardiomyocytes and the B6 chimeras showing acellular scar formation.

We next histologically examined the chimeric hearts and analysed the contribution of

(i) a dividing cardiomyocyte population using BrdU (the animals were given this from the time of injury to the end of the experiment);
(ii) a Y-positive cardiomyocyte population (derived from the stem cell population); and
(iii) the BrdU-positive and Y-positive doubly labelled overlap cardiomyocyte population. Were the BrdU-positive cells seen in the injury site in our previous study (Leferovich *et al.* 2001) derived from the bone marrow (these cells should then be doubly labelled with BrdU + Y)?

In these experiments, we examined four populations of mice, cryo-injured MRL and B6 chimeras and uninjured control MRL and B6 chimeras. In all of these mice, we found an unusually high number of BrdU-positive and Y-chromosome-positive cardiomyocytes throughout the heart. This is unlike what we have seen in normal uninjured mice and in cryo-injured mice as well that have not been lethally X-irradiated (at least for BrdU incorporation; Leferovich *et al.* 2001). Hence, lethal X-irradiation does induce an artefactual response and perhaps introduces an injury itself.

The data shown in figure 5 show the percentage of BrdU-positive, Y-positive or doubly positive cardiomyocytes in a whole heart cross-section as seen in figure 2. Though not shown here, the different sections of the heart analysed (the cryo-injured RV, the uninjured RV and the uninjured left ventricle) all showed large numbers of labelled cells. Also, the number of BrdU-positive cardiomyocytes is far greater than Y-positive cardiomyocytes in all of the groups.

Does cryo-injury affect the number of labelled cells? In the case of the MRL chimeras, the number of BrdU-positive cells is almost twice that of the uninjured chimera and is greater than the number of BrdU-positive cells in either of the B6 groups. Furthermore, in the MRL, the number of Y-positive cells is almost threefold greater in the injured versus the uninjured control. This is true not just in the injury site but throughout the heart. In the B6 chimeric hearts, the number of BrdU-positive or Y-positive cardiomyocytes does not change after cryo-injury. Thus, and the MRL heart reveals a response after cryo-injury that seems to be heart-wide and affects both the BrdU-positive as well as the Y-positive cells, whereas the B6 heart shows little difference with or without the cryo-injury.

Though BrdU-positive cells are clearly greater in number, are the Y-positive cells all BrdU positive? In fact, there is some overlap of these two populations but they are not in the majority. Thus, besides the bone marrow-derived stem cells that become cardiomyocytes, there is another population involved with cellular proliferation in the heart. It is not clear what is responsible for the BrdU-positive cells or their origin.

The Y-positive cells, introduced as bone marrow stem cells, must both enter the heart tissue and transdifferentiate. These cells may individually transdifferentiate into cardiomyocytes or fuse with a pre-existing cardiomyocyte as previously reported (Terada *et al.* 2002; Oh *et al.* 2003). We have not resolved what is responsible for these BrdU-positive cells, but they are clearly radioresistant cells. Are they dividing or is this just a demonstration of DNA

repair; is there also nuclear division or cellular division (Bettencourt-Dias *et al.* 2003; Urbanek *et al.* 2003)? And finally, the answer to the question we had attempted to address, the source of the BrdU-positive cardiomyocytes that fill the MRL injury site, is still unknown. It is, however, interesting that the response to injury leads to greater numbers of both BrdU- and Y-positive cardiomyocytes, possibly indicating increased growth or transdifferentiation signals in the MRL after injury.

Given the fact that Y-positive cardiomyocytes are found in the chimeric hearts, the next set of experiments examined the contribution of the bone marrow-derived cells to the healing phenotype. In this case, we employed F_1 bone marrow cells (F_1 mice are complete heart healers) injected into lethally X-irradiated nonhealer B6 mice. Thirty days after cryo-injury, histological sections of the heart revealed an injury site that looked like B6-type healing with an acellular scar (figure 6). This is significant in that stem cells do go to the heart and become cardiomocytes, but it appears that the radioresistant cells of the host are determining the healing phentoype. Another way of looking at this is to say that the stem cells do *not* contribute to the healing phenotype in this experimental system. Interestingly, these results are again similar to the chimeric earhole closure results carried out by a different group who showed that healer bone marrow into nonhealer mice still resulted in nonhealer chimeric mice (Kench *et al.* 1999).

From these results, we then have to conclude that there is a Y-positive cardiomyocyte population that may contribute to healing, but there is at least a second population of cells that are clearly involved and these may be the BrdU-positive population seen after injury.

8. CNS INJURY AND SCAR FORMATION

In addition to the ear and the heart, we have examined the ability of the MRL to heal CNS injuries. Recent experiments employing a cortical stab wound showed some similarities in MRL healing in the brain and other tissue. It was found that MRL mice suffered a more severe injury response than the control SW mice with more cell death but also with more BrdU-positive cells. There was also an enhanced inflammatory response and more widespread blood–brain-barrier leakage. Again, the MMP response was upregulated in the MRL; however, in the brain this response was quite transient. By day 14, however, both the MRL and the SW injuries looked the same (Hampton *et al.* 2004). It may be that the CNS has mechanisms to downregulate the MMP response, a potential key positive mediator of regeneration.

In a second area of interest, the spinal cord, we were able to test our hypothesis that scar tissue would lead to wound repair, but healing without the scar would result in a regenerative response. Using a minimal spinal cord transection injury, two models of spinal cord injury were developed (Seitz *et al.* 2002). In the first, a transection method was used in which the meninges and the main vessels were cut but the dislocation of the cut cord ends, the injury, and the scar was minimized. This was called the 'cut dura' model. In the second, the dura mater was left largely intact as well as the dorsal spinal vein. This was called the 'intact dura' model.

We found that compared with transections that are more invasive, cord itself in both of these cases. However, what proved quite impressive was that injury without scarring (the 'intact dura' model) led to mice that could functionally recover. And this was found to be the case in both the regenerating MRL and the nonregenerating B6 mouse (Seitz *et al.* 2002). Retrograde tracing using biocytin showed axonal growth and staining in the brainstem including the nucleus ruber and the cortex. Also, histological examination of the injury site showed extensive bridging across the transection with GAP-43-positive axonal growth cones, axonal extensions and astrocytes present throughout. This provides a *positive* model for adult regenerative CNS neurons.

Axonal regeneration has been reported for white matter tracts. Inoue *et al.* (1998) have shown that regeneration of the corticospinal tract can take place in young rats at the level of the brain stem. This ability of axons to show regeneration was also observed for optic nerve axons, to the extent that appropriate retinotopic maps were restored (Foerster & Holmes 1999). Inoue *et al.* (1998) suggested that the minimization of edema and infiltration is important for this to occur. In agreement with this, we have shown that upon reduction of infiltrates, a dramatic degree of regenerative ability of axons is present at lower thoracic levels of the spinal cord. This supports the view that at least some if not all neurons have an intrinsic capacity for axonal regeneration.

These spinal cord studies demonstrate that the scar plays a major role in blocking the regenerative response and that even in the C57BL/6 mouse, axonal regeneration occurs in its absence. Perhaps the same thing is true for humans. And perhaps, without the scar, the same type of healing and regeneration would be seen in the heart.

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GLOSSARY

BrdU: bromodeoxyuridine
CNS: central nervous system
FITC: fluorescein isothiocyanate
MMP: matrix metalloproteinase
MRL: murphy roths large
QTL: quantitative trait locus
RV: right ventricle
SW: Swiss Webster
TIMP: tissue inhibitor of MMP